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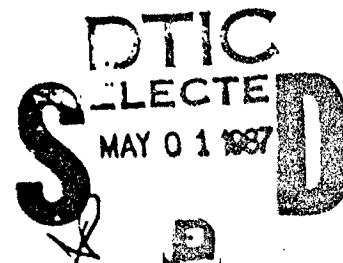
Regulation and Expression of a Key Proteolytic Enzyme in  
Bacterial Sporulation and Germination

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Final Technical Report

Peter Setlow

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		bacterial spore; radiation resistance; heat resistance; spore germination; proteolysis	
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Highlights of research findings in the past 3 1/2 years are: 1) The gene which we believe codes for the <u>Bacillus megaterium</u> spore protease has been cloned and its sequence is essentially complete; 2) We have generated deletion mutations of <u>B. subtilis</u> which lack genes for the three major small acid-soluble spore proteins (SASP). While all of these mutants grow and sporulate normally, mutations in two of these genes ( <u>sspA</u> and <u>sspB</u> loci) result in spores which are more heat sensitive than wild type spores, and much more sensitive to ultraviolet (UV) irradiation. This UV sensitive spore phenotype is cured by restoration of multiple copies of either the <u>sspA</u> or <u>B</u> genes; 3) UV irradiation of the mutant spores generated thymine dimers, in contrast to the spore photoproduct formed on comparable irradiation of wild type spores; 4) Findings 2 and 3 strongly suggest that SASP play a major causal role in spore UV resistance. <u>Keywords:</u>			
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## Final Technical Report

The objectives and abstract of studies to be undertaken as given in the initial grant application were as follows:

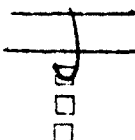
### Objectives

The overall objective of this research project is to gain a detailed understanding of the regulation of a specific B. megaterium spore protease - in particular the regulation of the enzyme's activity and synthesis. Specific objectives include: 1) Identification and characterization of the enzyme(s) which convert the inactive protease precursor P<sub>46</sub> to an active form of the enzyme (P<sub>41</sub>); 2) determination of the mechanism(s) whereby the enzyme(s) processing P<sub>46</sub> to P<sub>41</sub> is regulated; 3) determination, through analysis of P<sub>46</sub> to P<sub>41</sub> conversion in vitro, of the conditions in the developing forespore during P<sub>46</sub> to P<sub>41</sub> in vivo; 4) application of the knowledge gained in 3 to regulation of other key spore enzymes; 5) determination of the mechanisms which regulate the activity and processing of P<sub>41</sub>, such that these are only observed upon germination; 6) identification and characterization of the enzyme(s) which convert the protease from the P<sub>41</sub> form to the P<sub>40</sub> form; 7) cloning and sequencing of the spore protease gene; 8) placing the spore protease gene in an appropriate expression vector to allow production of large amounts of spore protease; 9) carrying out initial experiments to determine how the spore protease gene is regulated; and 10) mapping the protease gene in B. subtilis and isolation of deletion mutants in this gene.

The studies outlined in this proposal should provide new fundamental knowledge in three different areas. 1) They should give new information about the spore protease itself. 2) They should provide insight into the regulation of gene expression during sporulation; and 3) they should tell us how the enzymatic activity of the spore protease is regulated and thus in part how the enzymatic dormancy of a bacterial spore is regulated. The studies on the latter topic will almost certainly provide us with information concerning conditions inside the developing spore late in sporulation, and thus may provide new insight into the mechanisms of spore resistance to heat and ultraviolet irradiation.

### Abstract

The regulation of a specific bacterial spore protease from Bacillus megaterium will be investigated, both at the level of enzyme regulation and the level of gene expression. This enzyme is of interest because it is a sporulation specific gene product which is synthesized as an inactive precursor during sporulation and processed 2-3 hr later to a form which is active in vitro but not in vivo. Only upon initiation of spore germination does this enzyme act in vivo, initiating rapid degradation of a group of spore proteins which serve as an amino acid reserve, and which may also be involved in spore ultraviolet light resistance. The enzyme(s) and/or intracellular conditions involved in regulating the conversion of the protease from inactive precursor to active enzyme will be identified. Any activating or processing enzyme(s) will be studied in detail including its (their) mechanism of action and regulation. Potential mechanisms for regulating the protease's activity in vivo will also be investigated. These studies should provide information not only about regulation of the spore protease, but also may suggest mechanisms for regulation of other key spore enzymes, and these will be investigated. The gene for



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the protease will be cloned and analyzed, primarily to obtain production in an appropriate plasmid vector of large amounts of purified protease for use in studies of regulation of protease processing and activity. The cloned gene will also be used: a) to study the regulation of expression of the spore protease gene; and b) if time permits to isolate the B. subtilis spore protease gene and map it on the B. subtilis chromosome.

During the three and a half year period covered by this report we have made some progress on the objectives noted above, but it has been limited. More importantly has been the initiation of a new area of research that has given new insight into the mechanisms of spore resistance to heat and radiation. Highlights of these achievements are given below.

1) Cloning and analysis of the B. megaterium spore protease gene. The initial clone which we thought was the spore protease turned out to be a gene which coded for the major contaminant in the spore protease initially used as an immunogen to raise protease antibody. Since this clone was identified by antibody screening of a  $\lambda$ gt11 fusion library, isolation of a gene coding for a contaminant in the original immunogen was not surprising. This clone was sequenced, and comparison of the protein sequence in its open reading frame with national sequence data banks indicated that it coded for the protein analogous to heat shock protein 70 of eukaryotes and the E. coli Dnak protein.

Extracts containing this protein (produced in a plasmid expression vector) were used to adsorb out protease antisera to remove this spurious antibody. Use of this adsorbed serum in a rescreen of our expression library in  $\lambda$ gt11 allowed isolation of a number of other antibody positive clones which might contain the protease gene. Partial DNA sequence data is now available on several of these clones, and we are now awaiting some amino acid sequence data from the purified spore protease in order to definitively identify the spore protease containing clone.

2) Role of  $\alpha/\beta$  type small acid soluble spore proteins in spore resistance. Using recombinant DNA techniques we generated strains of B. subtilis with deletion mutations in the genes which code for one or both of the genes coding for two of the three major small, acid-soluble spore proteins (termed SASP- $\alpha$  and  $\beta$ ). These latter two SASP are almost 85% identical in primary sequence and are coded for by the sspA and sspB genes, respectively. The single and double deletions in these genes grew and sporulated normally, although the spores lacked the appropriate SASP. The  $\alpha^-$  and  $\alpha^- \beta^-$  spores were significantly more heat sensitive than were  $\beta^-$  and wt. spores (which showed identical resistance). However,  $\alpha^- \beta^-$  spores were still much more heat resistant than were vegetative cells of the wt. or  $\alpha^- \beta^-$  strains.

Spores of the  $\beta^-$  strain also showed similar UV resistance to that of wild type spores. However,  $\alpha^-$  spores were significantly more UV sensitive, with  $\alpha^- \beta^-$  spores being more UV sensitive still. Strikingly,  $\alpha^- \beta^-$  spores were more UV sensitive than were vegetative cells of the wt. or  $\alpha^- \beta^-$  strains, which showed identical UV sensitivity.

This data strongly implicate SASP, in particular SASP- $\alpha$ , in spore UV resistance, but the lack of effect of the  $\beta^-$  deletion was surprising, since SASP- $\alpha$  and  $\beta$  are almost identical proteins. This inconsistency was explained by quantitative analysis of levels of SASP  $\alpha$  and  $\beta$  in spores. SASP- $\alpha$  is normally present at a level 2-3 times that of SASP- $\beta$ , and in a  $\beta^-$  deletion

SASP- $\alpha$  levels rise  $\sim$  30-40%. Similarly, in an  $\alpha^-$  deletion SASP- $\beta$  levels rise  $\sim$  50%. However, in the latter strain the total level of this type of SASP ( $\alpha/\beta$ ) is not sufficient for full UV resistance, while essentially wild type levels of  $\alpha/\beta$  type SASP are present in the  $\beta^-$  spores.

This conclusion was further substantiated by the demonstration that the UV sensitive phenotype of  $\alpha^- \beta^-$  spores could be cured by introduction into this strain of either a single sspA gene or multiple sspB genes, the latter providing sufficient SASP- $\beta$ . This data thus suggests that any  $\alpha/\beta$  type SASP can provide spore UV resistance as long as sufficient SASP is present. Indeed, multiple copies of genes coding for minor SASP of the  $\alpha/\beta$  type also restored much of the UV resistance of  $\alpha^- \beta^-$  spores!

3) Photochemistry of DNA in wt. and  $\alpha^- \beta^-$  spores. Much work in other labs has shown that the major lethal DNA photoproducts produced by UV irradiation of growing bacteria, including *B. subtilis*, are the cyclobutane type pyrimidine dimers. In contrast, UV irradiation of dormant spores generates no cyclobutane dimers, but rather a thymine-thymine adduct, termed spore photoproduct. This spore photoproduct is formed in DNA with efficiency comparable to that of cyclobutane dimers, but is repaired much more efficiently thus accounting for the greater UV resistance of bacterial spores as compared to cells. Strikingly, UV irradiation of  $\alpha^- \beta^-$  spores produces about 1/2 as much spore photoproduct as in comparably irradiated wt. cells and 1/2 as much cyclobutane dimers as comparably irradiated vegetative cells. This production of the more lethal cyclobutane dimers upon UV irradiation of  $\alpha^- \beta^-$  spores explains their increased UV sensitivity, and implicates  $\alpha/\beta$  SASP as determinants of spore DNA photochemistry. While the exact mechanisms determining whether UV irradiation of DNA generates cyclobutane dimers or spore photoproduct are not known in complete detail, a major factor appears to be the level of DNA hydration. In general poorly hydrated or dry DNA gives spore photoproduct, while more hydrated DNA gives cyclobutane dimers. This would suggest that  $\alpha/\beta$  type SASP affect DNA hydration levels. While this is difficult to measure directly inside spores, we do know that spore core water contents (in terms of gm. of water per gm. dry wt.) appear identical in both wt. and  $\alpha^- \beta^-$  spores.

4) Lack of a role for the third major SASP, SASP- $\gamma$ , in spore resistance. We have cloned and sequenced the gene (termed sspE) which codes for the third major *B. subtilis* SASP (termed SASP- $\gamma$ ). SASP- $\gamma$  is quite different in primary sequence from the  $\alpha/\beta$  type SASP. We have also constructed an sspE gene deletion strain which grows and sporulates normally, and lacks all SASP- $\gamma$ . These mutant spores, as well as  $\alpha^- \gamma^-$  and  $\alpha^- \beta^- \gamma^-$  spores have been prepared; studies of their heat and UV resistance have indicated that SASP- $\gamma$  plays no significant role in the resistance of spores to these treatments.

#### Papers Published

1. Fliss, E.R., M.J. Connors, C.A. Loshon, E. Curiel-Quesada, B. Setlow, and P. Setlow, "Small, Acid-Soluble Spore Proteins of *Bacillus*: Products of a Sporulation-Specific, Multigene Family", In *Molecular Biology of Microbial Development* (J. Hoch and P. Setlow, ed.), p. 60-66, Amer. Soc. for Microbiol., Washington, D.C. (1985).

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3. Mason, J.M. and Setlow, P. "Evidence for an essential role for small, acid soluble spore proteins in the resistance of Bacillus subtilis spores to ultraviolet light", J. Bacteriol. 167:174-178 (1986).
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